PB81-184806

Modeling the Ames Test

(U.S.) Health Effects Research Lab.
Research Triangle Park, NC

15 Aug 80

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1. REPORT NO.	2.	3. RECI
EPA-600/J-80-062	JOURNAL ARTICLE	PB81-184806

4. TITLE AND SUBTITLE	5. REPORT DATE
Modeling the Ames Test	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S)	8. PERFORMING ORGANIZATION REPORT NO.
Andrew G. Stead, V. Hasselblad, John Creason, and Larry Claxton	
9. PERFORMING ORGANIZATION NAME AND ADDRESS	10. PROGRAM ELEMENT NO.
Biometry Division Health Effects Research Laboratory EPA-Research Triangle Park, N.C. 27711	A9RA1B 11. CONTRACT/GRANT NO.
12. SPONSORING AGENCY NAME AND ADDRESS	13. TYPE OF REPORT AND PERIOD COVERED
Office of Research and Development Health Effects Research Laboratory EPA-Research Triangle Park, N.C. 27711	14. SPONSORING AGENCY CODE EPA-600/11

15. SUPPLEMENTARY NOTES

Published In: Mutation Research 85:13-27, 1981

16. ABSTRACT

Despite the value and widespread use of the Ames test, little attention has been focused on standardizing quantitative methods of analyzing these data. In this paper, a realistic and statistically tractable model is developed for the evaluation of Ames-type data. The model assumes revertant colony formation at any dose follows a Poisson process while the mean number of revertants per plate is a nonlinear function of up to 4 parameters. An exponential decay term can be included in the model to adjust for toxicity. The resultant system of nonlinear equations is solved using a modified Gauss-Newton iterative scheme to obtain maximum likehood estmates of the model parameters. Significance of the key parameters is tested by fitting reduced models and using likelihood ratio tests. The model's performance is demonstrated on data from organic extracts of various environmental contaminants. Among the advantages of the proposed model are (1) no data is discarded in the parameter estimation process, (2) no arbitrary constants need to be added to zero counts or doses and (3) no mathematical transformation of the data is required.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

PRODUCT OF:
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS (This Report)	21. NO. OF PAGES
RELEASE TO PUBLIC	UNCLASSIFIED	
	20. SECURITY CLASS (This page) UNCLASSIFIED	22. PRICE

EPA Form 2220-1 (9-73)

Reprinted from

EPA-600/J-80-062
JOURNAL ARTICLE

ELSEVIER/NORTH-HOLLAND BIOMEDICAL PRESS, AMSTERDAM

Mutation Research, 85 (1981) 13–27
Elsevier/North-Holland Biomedical Press

MODELING THE AMES TEST

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(Received 5 February 1980)
(Revision received 12 August 1980)
(Accepted 15 August 1980)

Summary

Despite the value and widespread use of the Ames test, little attention has been focused on standardizing quantitative methods of analyzing these data. In this paper, a realistic and statistically tractable model is developed for the evaluation of Ames-type data. The model assumes revertant colony formation at any dose follows a Poisson process, while the mean number of revertants per plate is a nonlinear function of up to 4 parameters. An exponential decay term can be included in the model to adjust for toxicity. The resultant system of nonlinear equations is solved using a modified Gauss-Newton iterative scheme to obtain maximum likehood estimates of the model parameters. Significance of the key parameters is tested by fitting reduced models and using likelihood ratio tests. The model's performance is demonstrated on data from organic extracts of various environmental contaminants. Among the advantages of the proposed model are (1) no data is discarded in the parameter estimation process, (2) no arbitrary constants need to be added to zero counts or doses, and (3) no mathematical transformation of the data is required.

Due to its sensitivity in detecting mutagens, its economy both with respect to time and materials, and the well documented link between carcinogenicity and mutagenicity, the Ames test (Ames et al., 1973a,b, 1975) has become as basic to mutagenesis research as analysis of variance is to statistics. While geneticists continue to investigate new strains of Salmonella and other bacterial test systems as well as different types of activation systems under which these assays may be performed, surprisingly little attention has been focused on standardizing quantitative methods of analyzing these data.

In a typical assay, logarithmically spaced doses of the suspected mutagen are mixed with the tester strain, and the number of colonies reverting to histidine

prototrophy are counted. Results are usually reported as revertant colonies per plate for each dose of compound used. Various methods have then been employed for determining whether the compound produces positive results for a particular strain and metabolic activation type. As it is not our objective to critique these methods, we shall simply mention some of the analytic approaches with which we are familiar to illustrate their diversity.

In a review of 100 carcinogenic and non-carcinogenic synthetic organic chemicals, Commoner (1976) used the concept of a mutagenic activity ratio. This ratio is defined as the difference between experimental and control revertant colony counts divided by the average of all controls for a particular type of strain and microsomal preparation over all compounds tested. The experimental count was chosen from the dose level which maximized this ratio. Selecting the combination of strain, tissue, microsomal preparation, and concentration which yielded the highest mutagenic activity ratio, Commoner reported that, by classifying a compound with a ratio greater than 2.0 as positive and less than 2.0 as negative, both types of misclassification occurred at a rate of approx. 17%.

A more traditional dose–response approach to the analysis of this kind of data is being considered by the National Cancer Institute. As part of an elaborate project to make the results of all laboratories conducting cancer-related screening tests available in the form of a data bank, a statistical analysis package for Ames-type data is also provided (Chu, 1979). Using fairly extensive sets of criteria to exclude plate counts which are outliers or represent concentrations of the chemical for which the toxicity of the bioassay system precludes maximum mutagenic activity, simple linear regressions are performed on the remaining plate counts. Assuming a lognormal distribution of the plate counts, resultant regressions of the logarithms of the revertants on the logarithms of mutagen dose are tested for lack-of-fit. If there is no significant lack-of-fit, the results for a particular Salmonella strain and activation type are reported as positive if the linear trend is significant and at least one dose level's mean response exceeds an upper 95% empirical confidence limit established for solvent controls.

Weinstein and Lewinson (1978) advocate the use of multiple one-sided t-tests comparing the mean response at various $(n-1)$ dose levels of the suspected mutagen to the solvent control with m different concentrations of a liver enzyme preparation (S9). This results in $m(n-1)$ t-tests being performed. To ensure a low risk of false positives for this family of tests, these authors suggest the application of the Bonferroni inequality.

It is obvious from these methods that more effort needs to be directed towards incorporating the biological mechanisms involved in the Ames assay into the statistical analysis. One recent example is given by Haynes and Eckardt (1979) who have made some progress in this area by mathematically formalizing dose–response patterns for mutation induction data within the framework of single-event Poisson statistics. We propose in this paper one model for examining Ames-type data which is not only plausible biologically but is also statistically manageable.

Assay technique

All data appearing in this paper were generated using the standard plate incorporation technique described by Ames et al. (1975). 5 strains of *Salmonella typhimurium* were used: TA1535, TA1537, TA1538, TA100 and TA98. Specific mutant strains are exposed to a compound or mixture on agar plates deficient in histidine. Revertant colonies are then counted with an automatic colony counter. For the samples presented here, exposure was performed both in the presence and in the absence of a 9000 × *g* liver supernatant from male, Charles River, CD1 rats induced with Aroclor 1254. As a matter of convenience, the minimal histidine solution was incorporated into the base layer of the petri dish agar rather than into the overlay media. It has been demonstrated experimentally that this slight deviation from the Ames protocol does not effect the resultant plate counts.

All assays included both positive and negative controls. In the presence of S9, the positive control for all tests was 2-anthramine. In its absence, sodium azide was used for TA1535, methyl-*N*-nitrosoguanine for TA100, 2-nitrofluorene for TA1538 and TA98, and 9-aminoacridine for TA1537. When it was logistically possible, triplicate plate counts were used for all controls and at each level of the substance tested.

Model development

One major problem with the Ames assay is toxicity. Since the response to a given dose is reported as a count of revertant colonies, researches employing an analytic method similar to NCI's must decide what doses to retain before slope estimates are obtained. As the dose of the suspected mutagen is increased, the bioassay system's potential to produce revertants is hampered by a concurrent increase in toxicity. By recording revertants only, no estimate of the effect toxicity has in reducing the system's ability to produce mutant colonies is currently available using linear regression techniques. While for some mutation induction protocols concurrent assays can be performed to determine the number of cells surviving (and capable of forming a colony), this approach is not feasible using the plating technique. If approx. the same number of bacteria are placed on a plate to estimate surviving bacteria at each dose as are used to measure colonies, no accurate method of counting the survivors is currently available. On the other hand, if a smaller amount of bacterial solution is plated to facilitate cell counting, it can be argued that the measures of survivorship are based on different populations of cells than the colony counts.

Haynes and Eckardt (1979) mathematically characterized mutants produced in such systems as Ames as the net result of the independent effects of cell mutation and cell killing where the yield in mutants could be represented as the product of a power series in dose (mutation) and a negative exponential power series also in dose (killing). In a similar vein we hypothesized that when testing a non-mutagenic compound, the rate at which the culture becomes toxic, in terms of revertant colony counts, resembles an exponential decay curve. Letting t_i represent the proportion of bacteria available for mutation at dose x_i, we have

$$t_i = e^{-\beta x_i} \tag{1.1}$$

Count data has generally been treated as Poisson since Fisher (1958). Jorgenson (1961) recognized the application of multiple regression analysis with a Poisson process. We considered the follwing nonlinear Poisson model to describe revertant colony formation when assay toxicity was not a problem. Letting λ_i represent the mean number of revertant colonies per plate at dose x_i, we define the model as follows

$$\begin{aligned} \lambda_i &= \beta_0 \text{ for } x_i = 0 \qquad\qquad \beta_0 \geqslant 0 \\ &= \beta_0 + e^{\beta_1 + \beta_2 \log x_i} \text{ for } x_i > 0 , \end{aligned} \tag{1.2}$$

To adjust for toxicity effect, we define

$\lambda_i^* = \lambda_i t_i$, or more fully,

$$\begin{aligned} \lambda_i^* &= \beta_0 \text{ for } x_i = 0, \qquad \beta_0 \geqslant 0 \\ \lambda_i^* &= (\beta_0 + e^{\beta_1 + \beta_2 \log x_i})\, e^{-\beta_3 x_i} \text{ for } x_i > 0, \quad \beta_k \geqslant 0 \ (k = 0, 2, 3) \end{aligned} \tag{1.3}$$

where

β_0 = spontaneous reversion count,
β_1, β_2 = parameters which jointly describe the amount and rate of revertant colony formation with increasing dose, and
β_3 = rate at which system becomes toxic.

Still treating the counts as Poisson, we define the likelihood function

$$L = \prod_{i=1}^{d} \prod_{j=1}^{n_i} (e^{-\lambda_i^*} (\lambda_i^*)^{y_{ij}})/(y_{ij}!) \tag{1.4}$$

or log likelihood,

$$l = -\sum_{i=1}^{d} \sum_{j=1}^{n_i} (-\lambda_i^* + y_{ij} \log(\lambda_i^*) - \log(y_{ij}!) \tag{1.5}$$

where

y_{ij} = an independent observation of the Poisson mean, λ_i^*, for the jth replicate at dose x_i,
λ_i^* = defined as in 1.3,
d = number of doses including negative control, and
n_i = number of replicates at dose x_i.

Differentiating (1.5) with respect to each of the parameters, β_k, we obtain maximum likelihood equations for the β_k. Using iterative methods, we then estimate the parameters. While other iterative schemes may work equally well with nonlinear systems of equations, we encountered the best rate of meaningful convergence when using the modified Gauss-Newton method suggested by Hartley (1961) in conjunction with the chopping rule discussed by Gallant (1975).

Various tests of interest may then be performed. A goodness-of-fit test may be made to determine the validity of the Poisson assumption by treating the mean number of revertants at each dose as the expected count at that dose and the recorded counts as the observed. The resultant chi-square statistic will have degrees of freedom equal to the total number of plates minus the number of

dose levels (including negative control) in the assay.

When the data exhibits very little evidence of a toxicity effect, model (1.2) may sufficiently describe the dose—response pattern. By fitting both the 3-parameter (1.2) and 4-parameter (1.3) models to the data, we estimate their respective log likelihoods. Multiplying the appropriate difference of these estimates by −2.0, we obtain a statistic which is asymptotically chi-square with 1 degree of freedom. If this chi-square value is greater than 0.45 (the 50th percentile of the distribution with 1 degree of freedom), we retain (1.3) as the final model; otherwise, we choose (1.2).

By estimating the (log) likelihood obtained by fitting an all means model, i.e.

$$\lambda_i = \bar{y}_i (i = 1,2,...d) \tag{1.6}$$

we obtain some measure of the adequacy of the final model (analogous to a lack-of-fit test in a linear regression framework) by comparing the likelihood estimated from the all means model (1.6) with that estimated from (1.2) or (1.3). Depending on the final model selected, this likelihood ratio test produces a chi-square statistic with $(d-3)$ or $(d-4)$ degrees of freedom. If model (1.2) is considered the final model, a test of the mutagenic effect of the assay is made by comparing the likelihood estimated from fitting the reduced model

$$\lambda_i = \beta_0 (\beta_0 \geqslant 0) \tag{1.8}$$

with that estimated from fitting (1.2). This likelihood ratio test will produce a chi-square with 2 degrees of freedom. If model (1.3) is selected as the final model, the test of mutagenicity can be made by comparing the likelihood estimated from fitting the reduced model

$$\lambda_i = \beta_0 e^{-\beta_3 x_i} (\beta_0, \beta_3 \geqslant 0) \tag{1.9}$$

with that estimated from fitting (1.3). This likelihood ratio test will also produce a chi-square with 2 degrees of freedom. A test of the toxicity effect is made only in the event that the 4-parameter model is selected as the final model, and is based on the same chi-square statistic described earlier.

Model requirements

In fitting the 4-parameter model (1.3), at least 5 dose levels (including control) are necessary. If assay toxicity is negligible, the reduced model (1.2) can be used with as few as 4 dose levels. Otherwise, the test of model adequacy will not have any degrees of freedom. In either case a minimum of 2 plates per dose level should also be employed to obtain a meaningful goodness-of-fit test as well as to decrease the variance of the parameter estimates.

Initial estimates can be obtained using linear regression applied to adjusted observed counts at the non-zero doses. Equation (1.3), can be written

$$\lambda_i = \beta_0 e^{-\beta_3 x_i} + e^{\beta_1 + \beta_2 \log(x_i) - \beta_3 x_i} \tag{1.10}$$

Setting $x_i = 0$ in the first term on the right of (1.10)

$$\log(\lambda_i - \beta_0) = \beta_1 + \beta_2 \log(x_i) - \beta_3 x_i \tag{1.11}$$

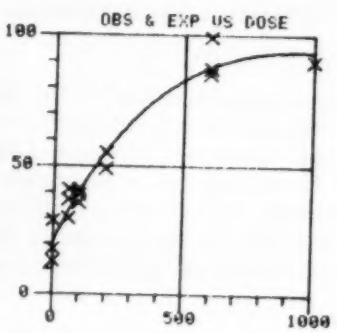
By first estimating β_0 from control information and then subtracting this estimate from the observed count at the respective dose, the parameters β_1, β_2 and

SAMPLE ID: MSER790856BMGS ACTIVATION: -
STRAIN: TA98 DATE: 08/21/79 TECHNICIAN: MJK

DOSE	UNITS	PLATE COUNTS			MEAN	S.D.
.00	UGS	29	13	18	20.00	8.19
3.00*	UGS	189	196	208	197.67	9.61
60.00	UGS	30	37	41	36.00	5.57
100.00	UGS	36	41	39	38.67	2.52
200.00	UGS	49	55	55	53.00	3.46
600.00	UGS	85	99	87	90.33	7.57
1000.00	UGS	90			90.00	.00

	B(0)	B(1)	B(2)	B(3)
ESTS.	20.489	-1.2629	.9618	.00893

TEST	CHI-SQUARE	DF	P	LOGL
POISSON	10.47	10	.4001	-50.1233
ADEQUACY	1.65	2	.4377	-50.9496
TOXICITY	3.24	1	.0717	-52.5718
MUTAGENICITY	44.36	2	.0000	-73.1293



SAMPLE ID: MSER790856BMGS ACTIVATION: +
STRAIN: TA98 DATE: 08/21/79 TECHNICIAN: MJK

DOSE	UNITS	PLATE COUNTS			MEAN	S.D.
.00	UGS	42	38	44	41.33	3.06
.50*	UGS	1259	1323	1216	1266.00	53.94
60.00	UGS	50	61	57	56.00	5.57
100.00	UGS	77	66	78	73.67	6.66
200.00	UGS	112	108	94	104.67	9.45
600.00	UGS	214	204	163	193.67	27.02
1000.00	UGS	169			169.00	.00

	B(0)	B(1)	B(2)	B(3)
ESTS.	41.922	-2.8712	1.4467	.00280

TEST	CHI-SQUARE	DF	P	LOGL
POISSON	12.01	10	.2843	-56.3413
ADEQUACY	1.19	2	.5526	-56.9345
TOXICITY	29.61	1	.0000	-71.7415
MUTAGENICITY	122.18	2	.0000	-110.0231

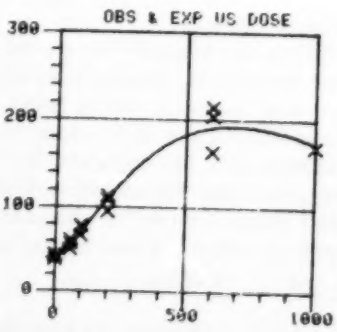


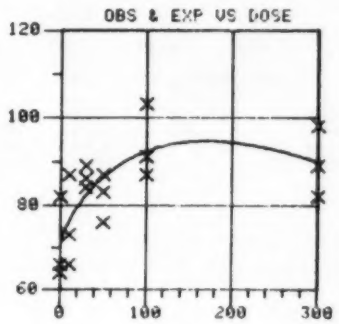
Fig. 1. Examples of the application of the nonlinear Poisson model to dose–response assays of environmental samples using TA98 without metabolic activation (top) and with metabolic activation (bottom).

SAMPLE ID: MSER790051NSNC ACTIVATION: -
STRAIN: TA100 DATE: 08/09/79 TECHNICIAN: PMM

DOSE	UNITS	PLATE COUNTS	MEAN	S.D.
.00	UGS	66 82 64	70.67	9.87
1.00*	UGS	406 420 485	439.67	40.77
10.00	UGS	66 73 87	75.33	10.69
30.00	UGS	89 84 86	86.33	2.52
50.00	UGS	76 83 87	82.00	5.57
100.00	UGS	87 103 91	93.67	8.33
300.00	UGS	89 98 82	89.67	8.02

	B(0)	B(1)	B(2)	B(3)
ESTS.	70.651	.1733	.7919	.00231

TEST	CHI-SQUARE	DF	P	LOGL
POISSON	9.61	12	.6503	-60.9979
ADEQUACY	1.36	2	.5068	-61.6776
TOXICITY	1.75	1	.1859	-62.5524
MUTAGENICITY	7.35	2	.0253	-65.3548



SAMPLE ID: MSER790056BMGS ACTIVATION: +
STRAIN: TA100 DATE: 08/21/79 TECHNICIAN: MJK

DOSE	UNITS	PLATE COUNTS	MEAN	S.D.
.00	UGS	131 104 115	116.67	13.50
.50*	UGS	947 950 923	940.00	14.80
60.00	UGS	218 203 228	216.33	12.58
100.00	UGS	271 261 233	255.00	19.70
200.00	UGS	346 360 344	350.00	8.72
600.00	UGS	420 372 371	387.67	28.01
1000.00	UGS	388	388.00	.00

	B(0)	B(1)	B(2)	B(3)
ESTS.	115.631	1.7796	.7358	.00187

TEST	CHI-SQUARE	DF	P	LOGL
POISSON	12.15	10	.2753	-64.9780
ADEQUACY	8.37	2	.0152	-69.1622
TOXICITY	26.86	1	.0000	-82.5942
MUTAGENICITY	291.58	2	.0000	-214.9509

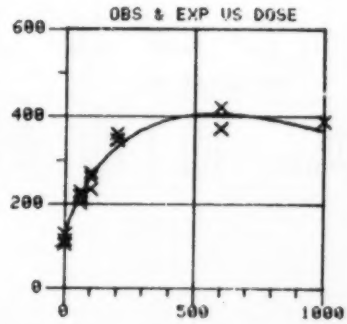


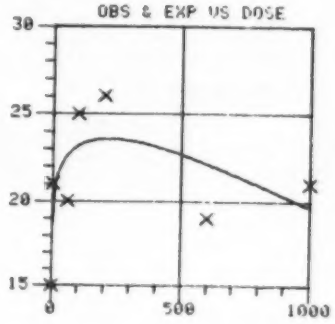
Fig. 2. Examples of the application of the nonlinear Poisson model to dose—response assays of environmental samples using TA100 without metabolic activation (top) and with metabolic activation (bottom).

SAMPLE ID: TAEB780502NSNC ACTIVATION: -
STRAIN: TA1535 DATE: 12/12/78 TECHNICIAN: LKS

DOSE	UNITS	PLATE COUNTS	MEAN	S.D.
.00	UGS	21 15	18.00	4.24
1.00*	UGS	307 306	306.50	.71
60.00	UGS	20	20.00	.00
100.00	UGS	25	25.00	.00
200.00	UGS	26	26.00	.00
600.00	UGS	19	19.00	.00
1000.00	UGS	21	21.00	.00

	B(0)	B(1)	B(2)	B(3)
ESTS.	17.952	.3429	.3249	.00846

TEST	CHI-SQUARE	DF	P	LOGL
POISSON	1.00	1	.3173	-17.5706
ADEQUACY	1.27	2	.5310	-18.2036
TOXICITY	.79	1	.3744	-18.5981
MUTAGENICITY	1.70	2	.4268	-19.0550



SAMPLE ID: GTDX790016NSNC ACTIVATION: +
STRAIN: TA1535 DATE: 07/06/79 TECHNICIAN: PMM

DOSE	UNITS	PLATE COUNTS	MEAN	S.D.
.00	UGS	15 15 9	13.00	3.46
3.00*	UGS	321 321 356	332.67	20.21
25.00	UGS	13 19 20	17.33	3.79
75.00	UGS	13 23 16	17.33	5.13
125.00	UGS	20 15 23	19.33	4.04
250.00	UGS	19 17 11	15.67	4.16
750.00	UGS	24 15 20	19.67	4.51
1250.00	UGS	17 12 16	15.00	2.65

	B(0)	B(1)	B(2)	B(3)
ESTS.	13.018	.9187	.1848	.00024

TEST	CHI-SQUARE	DF	P	LOGL
POISSON	13.44	14	.4921	-55.3974
ADEQUACY	2.51	3	.4734	-56.8525
TOXICITY	1.56	1	.2123	-57.6304
MUTAGENICITY	3.70	2	.1569	-58.7045

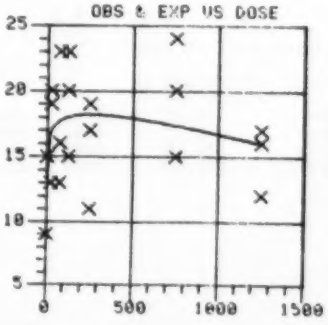


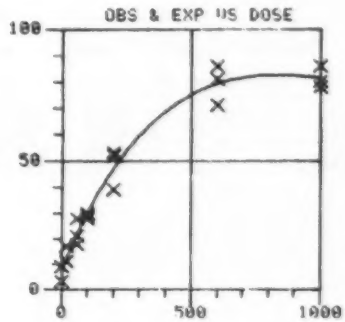
Fig. 3. Examples of the application of the nonlinear Poisson model to dose—response assays of environmental samples using TA1535 without metabolic activation (top) and with metabolic activation (bottom).

SAMPLE ID: GTOX790815NSNC ACTIVATION: -
STRAIN: TA1537 DATE: 07/11/79 TECHNICIAN: PMM

DOSE	UNITS	PLATE COUNTS	MEAN	S.D.
.00	UGS	3 9 9	7.00	3.46
100.00*	UGS	540 452 480	490.67	44.96
20.00	UGS	11 11 17	13.00	3.46
60.00	UGS	21 18 28	22.33	5.13
100.00	UGS	28 30 29	29.00	1.00
200.00	UGS	52 39 53	48.00	7.81
600.00	UGS	71 81 86	79.33	7.64
1000.00	UGS	86 80 78	81.33	4.16

	B(0)	B(1)	B(2)	B(3)
ESTS.	7.185	-1.0889	.9502	.00110

TEST	CHI-SQUARE	DF	P	LOGL
POISSON	12.14	14	.5950	-60.9145
ADEQUACY	.27	3	.9657	-61.0493
TOXICITY	12.27	1	.0005	-67.1836
MUTAGENICITY	126.79	2	.0000	-124.4429



SAMPLE ID: MSER790057BMGS ACTIVATION: +
STRAIN: TA1537 DATE: 08/22/79 TECHNICIAN: MJK

DOSE	UNITS	PLATE COUNTS	MEAN	S.D.
.00	UGS	14 14 12	13.33	1.15
3.00*	UGS	207 235 214	218.67	14.57
60.00	UGS	18 12 19	16.33	3.79
100.00	UGS	32 25 36	31.00	5.57
200.00	UGS	56 39 44	46.33	8.74
600.00	UGS	67 85 65	72.33	11.02
1000.00	UGS	66	66.00	.00

	B(0)	B(1)	B(2)	B(3)
ESTS.	12.495	-3.3002	1.3860	.00216

TEST	CHI-SQUARE	DF	P	LOGL
POISSON	10.60	10	.3891	-47.4741
ADEQUACY	4.52	2	.1043	-49.7342
TOXICITY	16.63	1	.0000	-58.0495
MUTAGENICITY	68.78	2	.0000	-84.1226

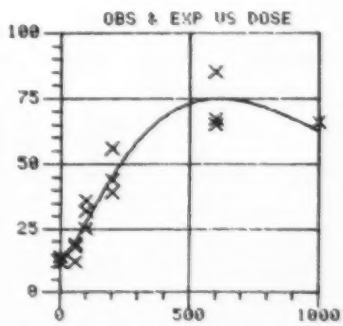


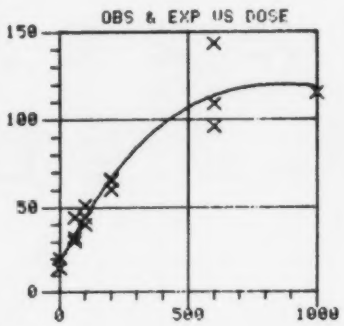
Fig. 4. Examples of the application of the nonlinear Poisson model to dose–response assays of environmental samples using TA1537 without metabolic activation (top) and with metabolic activation (bottom).

SAMPLE ID: MSEP790056BMGS ACTIVATION: -
STRAIN: TA1538 DATE: 08/21/79 TECHNICIAN: MJK

DOSE	UNITS	PLATE COUNTS	MEAN	S.D.
.00	UGS	20 20 14	18.00	3.46
3.00*	UGS	405 450 380	411.67	35.47
60.00	UGS	32 30 44	35.33	7.57
100.00	UGS	51 44 40	45.00	5.57
200.00	UGS	65 66 60	63.67	3.21
600.00	UGS	109 96 143	116.00	24.27
1000.00	UGS	115	115.00	.00

	B(0)	B(1)	B(2)	B(3)
ESTS.	18.289	-1.4037	1.0543	.00116

TEST	CHI-SQUARE	DF	P	LOGL
POISSON	16.44	10	.0878	-53.8781
ADEQUACY	.78	2	.6777	-54.2672
TOXICITY	7.55	1	.0060	-50.0417
MUTAGENICITY	84.48	2	.0000	-96.5068



SAMPLE ID: CMBX790028NSNC ACTIVATION: +
STRAIN: TA1538 DATE: 01/23/79 TECHNICIAN: LKS

DOSE	UNITS	PLATE COUNTS	MEAN	S.D.
.00	UGS	16 18	17.00	1.41
.50*	UGS	329 371	350.00	29.70
9.90	UGS	58 82	70.00	16.97
30.00	UGS	113 103	108.00	7.07
99.00	UGS	127 112	119.50	10.61
300.00	UGS	65 80	72.50	10.61

	B(0)	B(1)	B(2)	B(3)
ESTS.	16.902	2.9259	.5849	.00527

TEST	CHI-SQUARE	DF	P	LOGL
POISSON	7.19	5	.2070	-33.6186
ADEQUACY	.79	1	.3733	-34.0149
TOXICITY	39.47	1	.0000	-53.7496
MUTAGENICITY	195.51	2	.0000	-131.7687

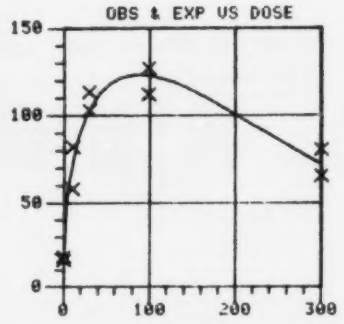


Fig. 5. Examples of the application of the nonlinear Poisson model to dose—response assays of environmental samples using TA1538 without metabolic activation (top) and with metabolic activation (bottom).

β_3 can be estimated by using a general linear model approach for the non-zero doses. We found these estimates provided very good starting values for any non-linear estimation procedure we used.

Some improvement in model performance could undoubtedly be achieved if outlier methodology were applied to these data. Very often, however, the application of such techniques raises more questions than it answers. Whenever the technican performing the assay is blind to the expected activity of test substance, the exclusion of a plate count or even a complete assay due to the technician's opinion that the count or assay has been arrived at through the use of faulty technique or procedures is certainly recommended.

Model application

Given the variety of strains and types of metabolic activation systems as well as test substances to which the model can be applied, it is very difficult to provide a comprehensive set of circumstances under which the model can be evaluated. Using test samples derived from various environmental contaminants, we have chosen situations which we feel allow the reader some objectivity in determining the model's feasibility.

The first set of examples (Figs. 1—5) illustrates samples which appear highly mutagenic (for most strains) initially but become toxic at high doses. Model behavior for each of the 5 tester strains is pictured with and without metabolic activation. All data shown do not necessarily arise from the same test substance.

The top half of each subfigure lists the recorded plate counts for each dose level tested. Positive control doses are asterisked and not included in the model fitting process. In the lower right corner of each figure a plot of the observed and model-predicted counts over the dose range tested appears. At the lower left, each subfigure contains parameter estimates for the final model and the results of various statistical tests described in the model development section.

The assay is considered positive if (1) the chi-square test of the Poisson assumption is not significant, (2) the fit of the final model is adequate when compared to the fit of the all means model via a likelihood ratio test, and (3) the likelihood ratio test for mutagenicity is significant. If either condition (1) or (2) is not met, the parameter estimates and other tests may still be valid but should be viewed with caution. Of the assays shown in Figs. 1—5, all except those pictured for TA100 (with activation) and TA1535 (with and without activation) would be considered positive under these criteria. The TA100 assay would be a qualified positive, and the two TA1535 assays would be classified negative.

In Figs. 6 and 7, the performance of the nonlinear Poisson model is compared with that of a simple linear regression model in which the log of the revertant colony count is regressed on the log of the sample concentration for 2 different samples. To avoid computational difficulties arising from zero doses and possible zero colony counts while using the latter model, an arbitrary constant of 1 was added to both counts and doses before taking logarithms. Even in the assay where there is no evidence of toxicity (Fig. 6), the simple linear regression model suffers from lack-of-fit while the 3-parameter model (1.2) describes the data quite well. In the assay where the effect of toxicity is visibly apparent

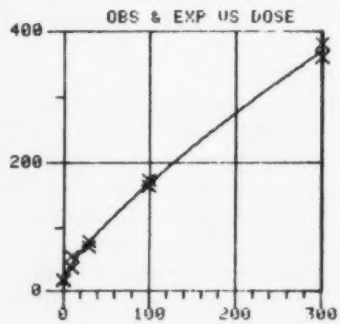
SAMPLE ID: CMBX790033NSNC ACTIVATION: +
STRAIN: TA1538 DATE: 01/23/79 TECHNICIAN: LKS

DOSE	UNITS	PLATE COUNTS	MEAN	S.D.
.00	UGS	16 18	17.00	1.41
.20%	UGS	329 371	350.00	29.70
9.90	UGS	37 55	46.00	12.73
30.00	UGS	70 79	74.50	6.36
99.00	UGS	164 174	169.00	7.07
300.00	UGS	380 360	370.00	14.14

	B(0)	B(1)	B(2)
ESTS.	17.396	1.5033	.7645

NO EVIDENCE OF TOXICITY

TEST	CHI-SQUARE	DF	P	LOGL
POISSON	5.02	5	.4135	-33.7202
ADEQUACY	.66	2	.7204	-34.0480
MUTAGENICITY	4313.76	2	.0000	-2190.9276



SAMPLE ID: CMBX790033NSNC
STRAIN: TA1538 ACTIVATION: +
DATE: 012379 TECHNICIAN: LKS

DOSE	UNITS	PLATE COUNTS	MEAN	S.D.
.00	UGS	16 18	17.00	1.41
.20%	UGS	329 371	350.00	29.70
9.90	UGS	37 55	46.00	12.73
30.00	UGS	70 79	74.50	6.36
99.00	UGS	164 174	169.00	7.07
300.00	UGS	380 360	370.00	14.14

AOV TABLE FOR REGRESSION

SOURCE	DF	SS	MS	F	P
REGRES	1	2.0072	2.0072	152.38	.0000
ERROR	8	.0659	.0132		
LOF	3	.0486	.0162	4.68	.0648
PURE	5	.0173	.0035		
TOTAL	9	2.0730	R-SQUARE =		.9682

B0 = 1.1814 B1 = .5263
SEB0 = .6091 SEB1 = .0426

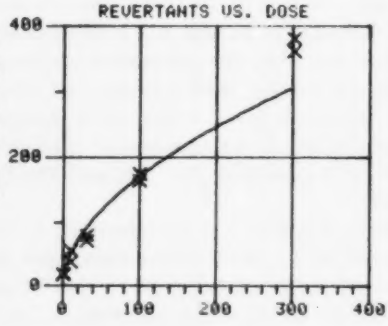


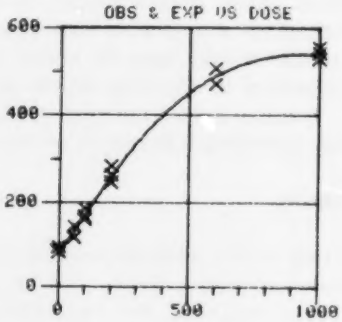
Fig. 6. Comparison of nonlinear Poisson model (top) to regression model of log count on log dose (bottom) for the same assay data.

SAMPLE ID: CMBX790013HSNC ACTIVATION: -
STRAIN: TA100 DATE: 01/16/79 TECHNICIAN: LKS

DOSE	UNITS	PLATE COUNTS			MEAN	S.D.
.00	UGS	83	95		89.00	8.49
1.00*	UGS	289	293		291.00	2.83
60.00	UGS	144	117	118	126.33	15.31
100.00	UGS	160	185	167	170.67	12.90
200.00	UGS	246	283	263	264.00	18.52
600.00	UGS	470	505	469	481.33	20.50
1000.00	UGS	542	554	528	541.33	13.01

	B(0)	B(1)	B(2)	B(3)
ESTS.	96.527	-.9574	1.2162	.00120

TEST	CHI-SQUARE	DF	P	LOGL
POISSON	11.44	11	.4073	-67.8830
ADEQUACY	1.81	2	.4040	-68.7092
TOXICITY	44.58	1	.0000	-90.9980
MUTAGENICITY	297.57	2	.0000	-217.4939



SAMPLE ID: CMBX790013HSNC
STRAIN: TA100 ACTIVATION: -
DATE: 011679 TECHNICIAN: LKS

DOSE	UNITS	PLATE COUNTS			MEAN	S.D.
.00	UGS	83	95		89.00	8.49
1.00*	UGS	289	293		291.00	2.83
60.00	UGS	144	117	118	126.33	15.31
100.00	UGS	160	185	167	170.67	12.90
200.00	UGS	246	283	263	264.00	18.52
600.00	UGS	470	505	469	481.33	20.50
1000.00	UGS	542	554	528	541.33	13.01

ADV TABLE FOR REGRESSION

SOURCE	DF	SS	MS	F	P
REGRES	1	1.0439	1.0439	40.50	.0000
ERROR	15	.2836	.0258		
LOF	4	.2720	.0680	64.42	.0000
PURE	11	.0116	.0011		
TOTAL	16	1.3275	R-SQUARE =		.7864

B0 = 1.7868 B1 = .2825
SEB0 = .6280 SEB1 = .0444

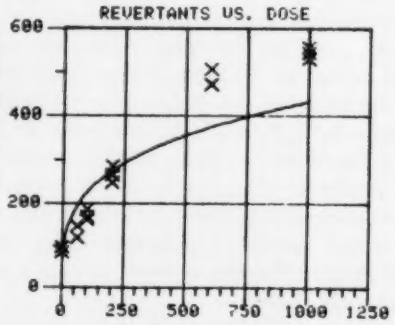


Fig. 7. Comparison of nonlinear Poisson model (top) to regression model of log count on log dose (bottom) for the same assay data.

(Fig. 7), the simple linear regression model fits the data very poorly while the 4-parameter model (1.3) does quite well. This is reasonable due to the nature of the models.

In the simple regression model, the rate at which the assay becomes mutagenic is assumed to be constant. A changing rate of mutagenicity with increasing dose can be handled quite easily, however, with the 3-parameter model (1.2). Furthermore, if toxicity is evident, gradual reductions in the mutagenic response at the higher doses can only be explained by the slope of the least squares straight line through the points with the simple linear regression model. Predicted values of the simple linear regression model will underestimate the number of revertants with increasing dose. Admittedly, the simple linear regression model would have fit better at higher doses had a constant other than 1 been added to the dose before taking logarithms. However, use of the nonlinear Poisson model entirely obviates such arbitrary decisions while simultaneously providing a better fit to the observed data.

Discussion

Fitting of the proposed model offers several advantages over a simple linear regression approach. First, no data is discarded in the parameter estimation process, thus relieving the experimenter of deciding which concentrations to exclude because toxicity overpowers the assay's colony-producing potential. Additionally, the nonlinear Poisson model avoids the need to add constants to the data to handle zero counts and doses. While adding small constants to all plate counts and doses simplifies computations, it does little to enhance the investigator's confidence in predicted colony formation at low doses since the choice of constants is somewhat arbitrary.

One major drawback to using the nonlinear model is reduced computational speed. The use of a simple linear regression approach requires at most one inversion of the incidence matrix while the nonlinear solution requires such an inversion during each iteration. On a computer such as the Univac 1110, however, the number of assays which could be analyzed in the same time frame by either method was not perceptibly different since output generation in either case is limited by the speed of the receiving graphics terminal.

We recognize that equations (1.2) and (1.3) could be written in other forms. For instance, if the formation of revertant colonies is assumed to be a linear phenomenon, β_2 could be set equal to 1 in both (1.2) and (1.3) resulting in 2- and 3-parameter models, resp. We have found, however, from examining literally hundreds of assays in which asymptotic confidence limits for each of the model parameters were computed, that quite often the confidence interval for β_2 did not contain 1. Consequently, the inclusion of the β_2 parameter makes these models flexible enough to characterize assays in wich colony formation is not strictly linear.

In the sense that predicted colony formation starts at spontaneous background level, and is allowed to increase with dose in a not necessarily linear fashion while simultaneously being attenuated by an exponentially decreasing function of dose to compensate for cell killing, the model described is directly related to the biological process exhibited in dose—response patterns for all

strains. Furthermore, the model has been developed within a statistical framework which allows significance testing of any mutagenic effect demonstrated by Ames assays using widely accepted methods.

Note on software development

The computer software necessary to implement the outlined model has already been developed and is operational on a Univac 1110. This software package produces printer plots or charts similar to those displayed in Figs. 1—7. While the graph portion of each figure uses "canned" plot producing routines supplied by a large graphics oriented corporation, the remaining analytical software has been made as machine-independent as possible. Other than the graphics' subroutines, the analysis package currently consists of 1 main program and 10 subroutines. All such modules have been written and compiled using ASCII Fortran. Information concerning this package may be obtained by contacting: Andrew G. Stead, Biometry Division, U.S. Environmental Protection Agency, Environmental Research Center, Research Triangle Park, NC 27711; telephone: (919) 541-3143 or (FTS) 629-3143.

Acknowledgements

The authors would like to thank Drs. Michael Symons and Stephen Nesnow, as well as the referees, for their helpful comments and suggestions concerning the content and style of the paper, and Carol Evans for her diligent efforts in the development of the data handling and retrieval aspects of the computerized system which supplied us the environmental assay data.

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